MICROCOLONY BRUCELLACIDAL TEST[1]

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Numerous methods have been described for determining in vitro bactericidal action of blood or serum. All of these methods involve some adaptation of the technique of mixing serum containing native or added complement with living cells, and after suitable incubation, plating samples of this mixture to determine survivors. The most notable differences in the methods utilized for this determination are evident in the methods of reporting results. Gengou (1899) and Neisser and Wechsberg (1901) employed the technique of mixing heat-inactivated test serum with a constant amount of added complement, and recorded results as the number of survivors in test and control (heated normal serum) preparations. Miles and Misra (1938) did essentially the same thing by recording the number of organisms destroyed by a given sample of fresh blood, whereas Huddleson et al. (1945) placed emphasis on the dilution of serum or plasma capable of killing a given number of organisms. Irwin and Berman (1950) used a bactericidal index based on the difference between the reciprocal of the highest bacterial control dilution showing growth and the highest bacterial test dilution showing little or no growth. When multiple dilutions of test sera are tested against multiple dilutions of the bacterial suspension to determine a bactericidal index for each serum dilution, the basis for Irwin and Berman's (1950) statement that the laboratory procedures required are almost laborious, becomes obvious. The roll tube technique of colony counting, introduced by Esmarch (1886), has failed to gain popularity partly because of the lack of suitable devices for handling multiple tubes. This was partially overcome in our laboratory by a spinning device, designed to handle three tubes simultaneously. A bactericidal antibody technique utilizing this device was developed and used by Bachman (1949). This technique has the advantage of simplicity and safety to the operator, since the live organisms are handled only once during the test procedure and the total mixture is incubated in the test container. Disadvantages inherent to this technique, however, are the relative difficulty of counting colonies, and the prolonged incubation (4 to 6 days) required before reproducible colony counts can be obtained. The technique for brucellacidal antibody testing described here decreases the time, equipment, and medium requirements of the other techniques with which it has been compared.

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MATERIALS AND METHODS

The microcolony technique of brucellacidal antibody titration utilized an antigen consisting of a live suspension of smooth, virulent *Brucella abortus* suspended in phosphate-tryptose buffer (0.2 M Na_2HPO_4, 125 ml; 0.2 M NaH_2PO_4, 125 ml; tryptose, 1 g; distilled water, 750 ml; pH 7.0 ± 0.2). This suspension was adjusted to a standard density on the Klett-Summerson photoelectric colorimeter, and then diluted with phosphate-tryptose buffer to a final concentration of approximately 10^5 cells per ml. One 3-mm (OS) diameter 26-gauge nichrome wire loop of a 1:10 dilution of this suspension when spot plated to 2-1 agar (Henry, 1933) gave 45 to 55 colonies, all located in an approximate 7-mm diameter area. The immune rabbit sera used in the test were obtained from animals having received two or more weekly, 0.2-ml, subcutaneous injections of a steam-killed *B. abortus* suspension containing 170 billion cells per ml. These sera were heated at 56 C for 30 min to destroy brucellacidal activity. Also, the normal brucellacidal activity was adsorbed from pooled normal rabbit serum, which was to be used as complement. Guinea pig complement will not function satisfactorily in this system (Shrigley and Irwin, 1937). The

adsorption was accomplished by treating a 19-ml sample of pooled normal rabbit serum with a 1-ml suspension of 4 billion steam-killed *B. abortus* at 0 C for 2 hr. These cells were obtained by suspending the growth from the surface of 48-hr tryptose agar cultures in 0.85% NaCl solution, and were killed by steaming at 100 C for 1 hr. The cells were washed twice with sterile saline to remove soluble antigenic substances which might interfere with the test (Shrigley and Irwin, 1937; Dingle, Fothergill, and Chandler, 1938).

The brucellacidal test was set up in sterile, capped tubes (3 by 5/8 in.) as follows:

Tube No.	*Test Preparation*
1	0.1 ml heated immune serum + 0.1 ml of complement + 0.1 ml of cell suspension. Additional test preparations containing dilutions of the immune serum may be included in the test series. The lowest possible final test dilution of the serum using this technique is 1:3.
	Control Preparations
2	0.1 ml of test serum + 0.1 ml phosphate-tryptose buffer + 0.1 ml cell suspension.
3	0.1 ml complement + 0.1 ml phosphate-tryptose buffer + 0.1 ml cell suspension.
4	0.2 ml phosphate-tryptose buffer + 0.1 ml cell suspension.

All preparations were incubated for 3 hr at 37 C, after which 0.7 ml of sterile phosphate-tryptose buffer was added to each preparation. Each preparation was then spot inoculated in quintuplicate to a delineated 2-1 agar plate by means of a 3-mm (OS) diameter 26-gauge nichrome wire bacteriological loop. After 40 hr at 37 C, the discrete microcolonies in each of the inoculated areas were counted with the aid of a broad field binocular microscope (30×) using the oblique lighting technique described by Henry (1933). Although the colonies had an average diameter of only 0.14 mm at this time, and therefore were virtually invisible to the naked eye, under suitable magnification they were readily countable. The over-all appearance was that of an entire conventional plate, containing mature microcolonies, as viewed without magnification. The results were expressed as the percentage of cells killed in the test preparations. The percentage was obtained by dividing the number of organisms killed (average controls minus average survivors) by the average number of colonies found in the the three control preparations.

TABLE 1

Brucellacidal activity of normal and immune rabbit sera tested by microcolony technique

Preparation No.*	Microcolony Counts (Quintuplicate)					Average Count	Per Cent Killed
1	0	0	2	0	1	0.6	99
2	49	54	56	60	58	55.4	0
3	0	0	0	0	0	0	100
4	51	65	45	61	49	All controls	
5	49	53	71	58	61	55.8	
6	57	59	52	53	55		

* 1 = Heated immune serum plus complement plus live cells (test preparation); 2 = heated normal serum plus complement plus live cells (test preparation); 3 = normal serum plus live cells (test preparation); 4 = heated immune serum plus buffer plus live cells (serum control); 5 = complement plus buffer plus live cells (complement control); 6 = buffer plus live cells (buffer control).

RESULTS

A typical protocol using the microcolony technique is given in Table 1. The immune serum (preparation 1) gave 99+% killing when compared to the average colony count of all control inoculations. Preparation 2 (Table 1) shows that rabbit serum complement, after adsorption with *B. abortus*, does not reactivate the normal brucellacidal activity of heated rabbit serum. This contrasts with the observations of Irwin and Berman (1950) in their use of normal bovine serum.

Table 2 presents typical results obtained with this microcolony brucellacidal antibody testing procedure using adsorbed and unadsorbed immune rabbit sera. These results illustrate the commonly observed Neisser-Wechsberg (Neisser and Wechsberg, 1901) antibody excess zone of inhibition observed with such immune sera. The antibody excess zone correlated with the agglutinin titers of the antisera, and was re-

TABLE 2

Effect of specific adsorption of antisera to Brucella abortus antigens upon the brucellacidal and agglutinating activity of these antisera

B. abortus Antisera (Anti-)	*B. abortus* Adsorbing Antigen	Brucellacidal Activity* per Dilutions of Antiserum:					Agglutinin Titer (1:—)
		1:3	1:12	1:48	1:192	1:768	
Intact cell	None	0	33	96	99	99	1,280
Intact cell	Intact cell	100	100	93	51	27	10
Sonic disintegration†	None	0	82	99	100	93	690
Sonic disintegration†	Sonic disintegration†	99	93	29	—	2	10
Intact cell	None	0	36	79	98	100	640
Intact cell	Phenol extraction‡	100	100	99	93	93	20

* Per cent killed as compared to controls.

† Steam killed cells subjected to sonic disintegration in a Raytheon magnetostriction oscillator for 2 hr.

‡ The phenol extraction technique of Paterson, Pirie, and Stableforth (1947).

versed by adsorption with any of the 3 types of abortus antigens used as adsorbing agents. Efforts to find a *B. abortus* haptene that would accomplish this reversal were unsuccessful.

DISCUSSION

Extensive testing of this microcolony technique for determining brucellacidal activity in immune sera has indicated a reproducibility equal to that of other techniques described in the literature. Results showing a 10% or greater deviation of the average colony counts of the test preparation compared to those of the controls are significant. The quintuplicate spot inoculation method and the 10% average deviation were arrived at by determining the average deviation of quintuplicate counts selected at random from 100 spot inoculations made from a representative sample. The average deviation thus obtained is within the limits of experimental error.

The relatively short incubation time, combined with the reproducibility, economy, and simplicity of this testing procedure provides a satisfactory means for determining brucellacidal activity in normal and immune sera.

SUMMARY

A microcolony technique which affords a simple and accurate means of testing in vitro for brucellacidal antibodies is described. The technique was employed to demonstrate that rabbit serum complement, after adsorption with *Brucella abortus*, does not reactivate the normal brucellacidal activity of heated rabbit serum. The method was also used to demonstrate the reversal of the antibody excess zone of inhibition of brucellacidal activity by specific adsorption of antisera.

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